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Review

Thermozymes: Adaptive strategies and tools for their biotechnological applications

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ABSTRACT

In today's scenario of global climate change, there is a colossal demand for sustainable industrial processes and enzymes from thermophiles. Plausibly, thermozymes are an important toolkit, as they are known to be polyextremophilic in nature. Small genome size and diverse molecular conformational modifications have been implicated in devising adaptive strategies. Besides, the utilization of chemical technology and gene editing attributions according to mechanical necessities are the additional key factor for efficacious bioprocess development. Microbial thermozymes have been extensively used in waste management, biofuel, food, paper, detergent, medicinal and pharmaceutical industries. To understand the strength of enzymes at higher temperatures different models utilize X-ray structures of thermostable proteins, machine learning calculations, neural networks, but unified adaptive measures are yet to be totally comprehended. The present review provides a recent updates on thermozymes and various interdisciplinary applications including the aspects of thermophiles bioengineering utilizing synthetic biology and gene editing tools.

1. Introduction

Enzymes are biocatalysts having high catalytic power and substrate/reaction specificity. They are sought after molecules for industrial processes as their application leads to efficient generation of pure stereoisomers, fewer side reactions, easier separation of products, and lower environmental burden. Their use is limited by the fact that most of the enzymes work under ambient physiological conditions and get inactivated during industrial processes operational under harsh conditions. This necessitates the search for enzymes able to withstand extreme operational conditions. Enzymes from microorganisms growing at elevated temperatures are termed as thermozymes and considered as ideal candidates for catalytic processes operating at high temperatures. Process operations at elevated temperatures have several benefits *viz.* faster reaction rate, increased solubility of substrates, decreased viscosity, and elimination of microbial contamination (Gomes and Steiner, 2004). Owing to their significance, due focus has been paid on their research and this reflects from many reviews and articles highlighting their significance and benefits (DeCastro et al., 2016; Elleuche et al., 2014; Sarmiento et al., 2015; Sinha and Khare, 2013; Wohlgemuth et al., 2018).

Thermozymes have found use in diverse sectors not limiting to biofuel, food processing, pulp and paper, detergent, medicine, and nutraceuticals. One cannot have a better example for application of thermozymes than Taq polymerase and other subsequently developed thermophilic DNA polymerases used in Polymerase Chain Reaction (PCR), which revolutionized the area of genetic engineering. Advent of protein engineering field and sourcing of newly discovered thermozymes from novel isolates has expanded the repository of these enzymes. Some of the thermozymes have also shown stability and activity under other harsh conditions such as low pH, high pressure, and presence of salts and can be deemed suitable for applicability under polyextreme conditions. Further, stability in denaturants such as detergents and organic solvents made them suitable for organic synthesis (Atomi, 2005). Present review encompasses current trends in research on thermozymes.

2. Thermophiles and hyperthermophiles

Thermophiles are the class of organisms attuned to grow at elevated temperatures. Depending upon the range of temperature of their growth, they have been broadly divided into moderate thermophiles

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(50–60 °C), extreme thermophiles (60–80 °C), and hyperthermophiles (> 80 °C). The defined temperature range for above classes of thermophiles is differently indicated by various researchers. They have been isolated from natural environments such as geothermal areas, volcanic regions, hot springs, deep-sea hydrothermal vents, solar heated soils, geothermally heated oil, and petroleum reserves in addition to man-made environments like acid mine drainage, compost piles, and waste treatment plants (Mehta and Satyanarayana, 2013).

Observation and study on thermophiles started as early as 1960s with investigation of Yellowstone natural park (USA). In late 1970s and early 1980s, finding of microbial life in deep sea thermal vents and volcanic regions started (Frock and Kelly, 2012). During that period, *Sulfolobus acidocaldarius* was the organism with highest growth temperature of 85 °C (Brock et al., 1972). The first hyperthermophile isolated was *Methanothermus fervidus*, a novel rod shaped methanogen able to grow at 97 °C (Stetter, 2006). Gradually more researches led to isolation of additional thermophiles and the extreme temperature boundary for existence of life was redrawn with organisms like *Methanopyrus kandleri* being able to survive up to autoclavable temperature of 122 °C (Stetter, 2011). Thermophiles are mainly represented in prokaryotic domain of bacteria and archaea along with some eukaryotes which are able to grow up to 60 °C. Thermophiles are reported in bacterial genera of *Thermus*, *Bacillus*, *Clostridium*, *Rhodothermus,* and *Thermoanaerobacter* along with archaeal genera of *Methanobacterium* and *Thermoplasma*. *Aquifex*, *Thermotoga,* and *Hydrogenobacter* are bacterial genera from which species of hyperthermophiles are reported. However, hyperthermophiles are mainly reported from archaea and some of the representative genera are *Methanococcus*, *Methanothermus*, *Archaeoglobus*, *Pyrococcus*, *Pyrodictium*, *Pyrolobus*, *Sulfolobus*, *Thermococcus,* and *Thermoproteus* (Gomes and Steiner, 2004; Imanaka and Atomi, 2002). Amongst the variety of thermophilic isolates, three archaeal isolates of *Pyrococcus furiosus* (T_{opt} 98–100 °C), *Thermococcus kodakarensis* (T_{opt} 85 °C), *Sulfolobus solfataricus* (T_{opt} 80 °C), and *Thermotoga maritima* (T_{opt} 80 °C) from bacterial domain served as model organisms for studies to comprehend the basis of existence of life at high temperature (Frock and Kelly, 2012). Research interest in thermophiles originates from the curiosity to comprehend cell and protein adaptations at temperatures beyond boiling point of water and possibility of their applications. Regarding adaptability of their cells, shorter genome size; low GC content; saturated fatty acids in cell membrane to maintain rigidity at high temperature; chaperones and chaperonins for folding of macromolecules and reverse gyrase to protect DNA form some unique features (Gomes and Steiner, 2004; Urbieta et al., 2015). Some typical thermophiles and hyperthermophiles along with corresponding thermozymes are listed in Table 1. It is obvious from the list that a range of thermozymes from thermophiles have diverse biotechnological applications.

3. Thermozymes: structural and functional adaptations

Researchers have always been interested to decipher the structural features imparting stability to thermozymes at high temperature. This curiosity emanates from academic viewpoint as well as to have an idea to rationally design existing mesophilic enzymes for enhanced thermal stability by protein engineering. A range of factors contribute to thermostability in proteins and it varies from protein to protein. A single factor cannot be accounted for thermal stability and despite extensive research in this area a clear understanding to formulate a theory for protein thermostability is still elusive. Combinations of structural features impart thermostability in thermozymes; these features are discussed in following sections.

3.1. Intrinsic factors

3.1.1. Molecular interactions

Intrinsic factors can be more of molecular interactions such as

Table 1
Thermophiles/hyperthermophiles and corresponding thermozymes.

Microorganisms	Thermophile/hyperthermophile	Reported thermozymes	Temperature optima and stability	Possible industrial application (s)	Reference(s)
Bacillus amyloliquifaciens	Thermophile	α-Amylase	T_{opt} 70 °C (pH_{opt} 7.0); $T_{1/2}$ 6 h at 90 °C	Starch hydrolysis; baking; oligosaccharide synthesis	Kikani and Singh (2011)
Methanococcus jannaschii	Hyperthermophile		T_{opt} 120 °C (pH_{opt} 5.0–8.0); $T_{1/2}$ 50 h at 100 °C		Kim et al. (2001)
Thermotoga thermarum	Hyperthermophile	Xylanase	T_{opt} 95 °C (pH_{opt} 7.0); $T_{1/2}$ 60 min at 90 °C	Paper bleaching	H. Shi et al. (2013)
S. solfataricus	Hyperthermophile		T_{opt} 90 °C (pH_{opt} 7.0); $T_{1/2}$ 47 min at 100 °C		Cannio et al. (2004)
Meiothermus ruber	Thermophile	Protease	T_{opt} 100 °C; Stable at 80 °C	Detergents; baking; brewing	Kataoka et al. (2014)
Coprothermobacter proteolyticus	Thermophile		T_{opt} 85 °C (pH_{opt} 8.0); Stable at 80 °C		Toplak et al. (2013)
Rhodothermus marinus	Thermophile	Chitinase	T_{opt} 70 °C (pH_{opt} 4.5–5.0); $T_{1/2}$ 3 h at 90 °C	Chitin modification in food; pharmaceutical as health products	Hobel et al. (2005)
Thermococcus chitonophagus	Hyperthermophile		T_{opt} 70 °C (pH_{opt} 7.0); $T_{1/2}$ 60 min at 120 °C		Andronopoulou and Vorgias (2003)
Thermoanaerobacter thermohydrosulfuricus	Thermophile	Lipase	T_{opt} 75 °C (pH_{opt} 8.0); Stable at 70 °C	Biofuel; cosmetics; detergents; transesterification	Royter et al. (2009)
Bacillus thermoleovorans	Thermophile		T_{opt} 70–75 °C (pH_{opt} 7.5); $T_{1/2}$ 60 min at 60 °C		Lee et al. (1999)
Dictyoglomus thermophilum	Thermophile	Cellulase (endoglucanase)	T_{opt} 50–85 °C (pH_{opt} 5.0); $T_{1/2}$ 336 h at 70 °C	Biomass saccharification; textile; detergent	R. Shi et al. (2013)
Thermotoga neapolitana	Hyperthermophile	(endocellulases)	T_{opt} 106 °C (pH_{opt} 6.0–6.6); $T_{1/2}$ 130 min at 106 °C		Bok et al. (1998)
Thermus thermophilus	Thermophile	Pullulanase	T_{opt} 70 °C (pH_{opt} 6.5); $T_{1/2}$ 2 h at 80 °C	Starch saccharification; prebiotics; resistant starch; bread making	Wu et al. (2014)
T. kodakarensis	Hyperthermophile		T_{opt} 95–100 °C (pH_{opt} 3.5); $T_{1/2}$ 45 min at 100 °C		Ahmad et al. (2014)

hydrophobic, ionic, hydrogen bonds, disulphide bonds, and metal binding in addition to compact and rigid structure resulting from increased packing, shortening and stabilization of loops, and stability of α-helix (Li et al., 2005). Ionic interaction is a significant factor contributing to thermal stabilization of proteins. In general, proteins from thermophiles have greater content of charged amino acids such as glutamate, lysine, and arginine as compared to mesophiles (Liang et al., 2005). Greater electrostatic interactions resulting from salt bridges and ionic networks are important contributor to stability of proteins at high temperature (Karshikoff and Ladenstein, 2001; Nakayama et al., 2007). An optimized electrostatic interaction by increased formation of salt bridges is a key factor in themostabilization of hyperthermophilic proteins. This characteristic is less manifested in thermophilic proteins and is absent in mesophilic proteins (Karshikoff and Ladenstein, 2001). Ion pairs in thermostable proteins are found to be clustered together in networks. The arrangement of ion pairs is considerable for stability as these networks role to stability is greater than the sum total of individual ionic bond (Littlechild et al., 2013). Hydrogen bond is also a contributor in thermostability of thermophilic proteins and it occurs not only in the polypeptide chain but also with the neighboring aqueous medium. Charged-neutral hydrogen bonds have been found to impart thermostability to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes (Littlechild et al., 2007). Concealing hydrophobic residues within the core of protein during folding has foremost role in stability. The resulting hydrophobic interactions have significant contributions to thermostability of proteins (Goodenough and Jenkins, 1991). Thermostability of protein is directly correlated to total hydrobhobicity. A methyl group burial in core during protein folding accounts for stability increase by 1.3 kcal/mol (Pace, 1992). Augmentation in *Hydrogenobacter thermophilus* cytochrome c552 hydrophobicity by removal of hydroxyl group from protein core by substitution of a tyrosine by phenylalanine led to increase in melting temperature (Tm) by 6 °C (Takahashi et al., 2006). Earlier disulfide bonds were thought not to be involved in thermostabilization as cytosolic environment is reducing and added to this, high temperature leads to reduction of this bond. This notion was contradicted by various studies establishing role of this bond in stability of protein from thermophiles. *Bacillus pumilus* ML413 catalase thermostability and catalytic efficiency improved after introduction of disulphide bond. A single disulphide bond formation in this case led to increase in half-life at 60 °C by 40 min and catalytic efficiency was improved by 40% (Samson et al., 2018). Beeby et al. (2005) established the genomic evidence of disulphide bond abundance in proteins from diverse thermophiles. In this study protein disulfide oxidoreductase was found to be key enzyme associated with formation of intracellular disulphide bonds in thermophiles. Metal ions though not much discussed regarding stability of thermozymes, is another important component contributing to enzyme activation and stability. It has been ascertained that Mn^{2+} ions in case of *Bacillus licheniformis* xylose isomerase (Vieille et al., 2001) and Ca^{2+} in *Clostridium thermocellum* cellobiohydrolase confer thermal stability (Kataeva et al., 2003).

Amino acid compositions in primary structure of protein dictate much of molecular interactions and conformations. Comparison of amino acid compositions of thermophiles and mesophiles has revealed that proteins of thermophiles have amino acid bias to enhance stability under extreme temperature conditions. Some generalized features of amino acid composition in thermophilic proteins are additional charged residues, increase in residue hydrophobicity, less of uncharged polar residues, and more of aromatic residues (Zhou et al., 2008). As pointed out earlier, thermophilic proteins have more of charged residues resulting in greater ionic interactions to stabilize the structure. Substitution of Gly to Ala, Ser to Ala, Ser to Thr, Lys to Arg, and Asp to Glu have been observed in thermozymes (Argos et al., 1979). These substitutions contribute to thermostability by increasing internal hydrophobicity and decreasing flexibility of protein. Thermolabile amino acids present in thermophilic proteins are protected from degradation by conformation of structure which shields it from water (Vieille and Zeikus, 2001).

3.1.2. Molecular conformations

Conformational strength of thermozymes depends upon two contrasting features, flexibility, for the catalytic function of the enzyme, and rigidity, for conformational stability. Thermozymes are rigid compared to mesozymes and this feature protects them from deactivation at high temperature. Superior rigidity protects protein from proteolytic degradation, denaturation by chemicals, and unfolding at elevated temperature. Factors such as α-helix stabilization, electrostatic interactions, disulphide bridges etc. contribute to protein rigidity (Bruins et al., 2001; Li et al., 2005). Comparison of 20 complete genomic sequences has revealed that thermophilic proteins are shorter than their mesophilic counterparts. Further, sequence alignments of thermophilic proteins disclosed that exposed loops regions are deleted in their sequences (Thompson and Eisenberg, 1999). Loops are considered to be starting point for thermal denaturation hence their shortening adds to protein stability. The protein flexibility is also reduced by additional stabilizing interactions such as ionic interactions (Daniel et al., 2008). Rigidify flexible sites (RFS) approach by predicting flexible sites of proteins and then rigidifying it is an effective strategy to enhance thermostability (Yu and Huang, 2014).

As discussed earlier, increased hydrophobic interactions in thermozymes result from burial of hydrophobic residues in protein core. This factor also leads to increased packing with better van der Walls interactions and hence improved thermostability. Increased proportion of alanine, isoleucine, and proline is known to help in tighter packing of hydrophobic core and provide strength to loops of protein. Compactness of thermozymes is also attained by a combination of factors such as absence of cavities, loops shortening, and optimized packing of side chains in the core of protein (Corazza et al., 2006; Russell et al., 1997). α-Helix stabilization by having residues of high helical propensity and stabilizing interactions between domains and subunits such as formation of oligomers are also some of the intrinsic factors leading to increased thermostability (Bruins et al., 2001; Li et al., 2005).

3.2. Extrinsic factors

Various extrinsic factors such as high protein concentration, substrate, activators, cofactor, and specific salts are well known to stabilize proteins, be it thermophilic or mesophilic. Environmental condition of high pressure has been found to induce stability in hyperthermophilic proteins. This consequence from the fact that many hyperthermophilic environments are also high pressure one, hence proteins are adapted under both extreme conditions. Salts also stabilize proteins either by specific interaction of metal ions or by salting effect affecting the water activity (Vieille and Zeikus, 2001). Osmolytes though well known to be produced by halophiles to maintain osmotic balance under high salt conditions are also produced by thermophiles, when grown above optimum growth temperature. Osmolytes such as cyclic-2, 3-bisphosphoglycerate, diglycerol phosphate, mannosylglycerate, and di-myo-inositol-phosphate are identified to be produced in response to elevated temperature (Empadinhas and da Costa, 2006). Several studies have established that these osmolytes act as thermostabilizers of various thermozymes (Daniel et al., 2008; Littlechild et al., 2013). Osmolytes, glycine and sorbitol have been found to improve the kinetic properties and thermostability of *Lepidium draba* peroxidase (Sarvandi-Dehghanpoor et al., 2018). Other environmental aspects such as chemical cross-linking, immobilization, and glycosylation can also affect thermostabilization (Bruins et al., 2001). Proteins from thermophiles use a combination of above different factors for thermostabilization but none of the above feature can be accounted as universal.

On the other hand, distinction of thermophilic proteins from mesophilic ones has been a challenging work in order to design stable proteins. Study on discrimination between mesophilic and thermophilic proteins have used machine learning techniques that includes various methods based on neural networks, logistic functions, support vector

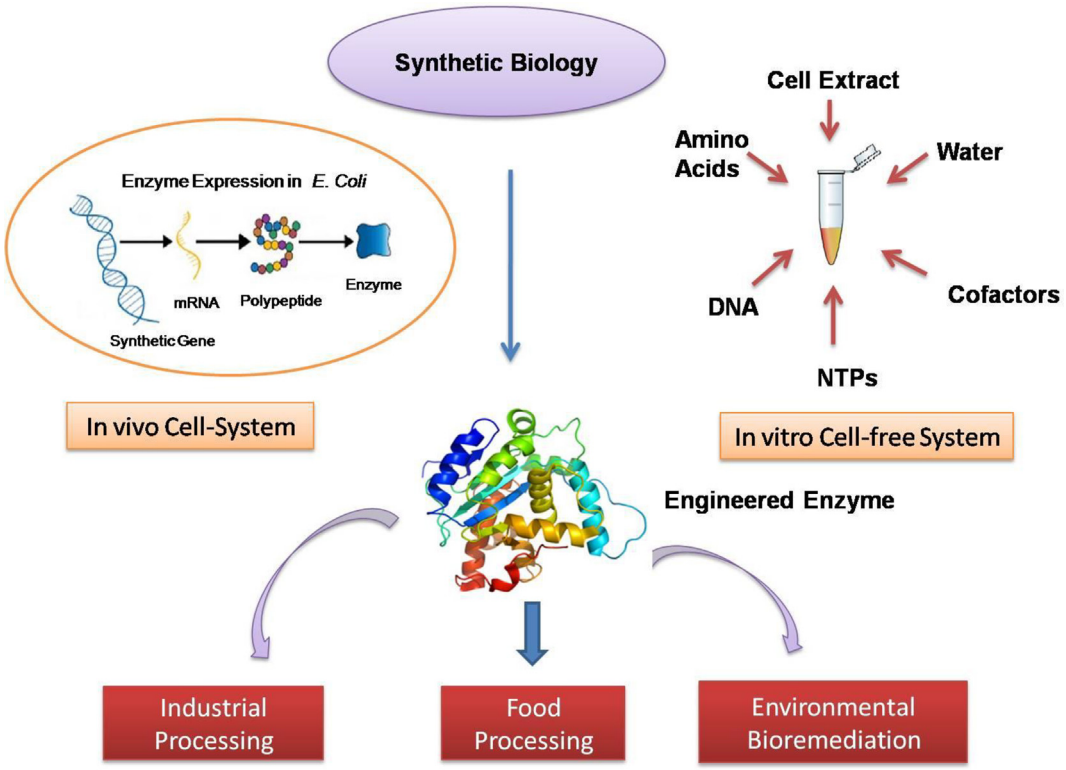

Fig. 1. Synthetic biology approaches for production of thermozymes for different applications.

machines, regression analysis, nearest neighbor methods, meta learning, etc. Studies have suggested that neural network based method is more successful in discriminating mesophilic proteins from that of thermophilic proteins. Machine learning algorithms have also been accessed to determine the most significant attributes that contribute to stability of protein at elevated temperatures (Gromiha and Suresh, 2008; Lakijadeh et al., 2011).

4. Synthetic biology for enzyme engineering

The advances in synthetic biology tools has provided new platform for enzyme engineering to increase the thermostability (Fig. 1). Recent studies demonstrated that thermozymes possesses extensive hydrogen bond interactions and aromatic clusters at N- and C-termini that increase their rigidity (Liu et al., 2018). So, could an enzyme be modified into thermozymes by incorporating these changes at specific places? The conventional enzyme engineering approach such as site-directed mutagenesis using recombinant DNA technology is widely used due to well studied and optimized protocols. Many enzymes are engineered using this conventional approach to modify different properties such as to improve kinetic and catalytic properties, to increase the stability of enzyme including thermostability, to inhibit allosteric regulation, and to enhance substrate specificity (Sharma et al., 2018). But certain limitations like low success rate, high cost, and time consuming process limits its usability.

Synthetic biology is mainly focused to write new genetic information, thereby help in designing non-natural genes and enzymes. It is becoming increasingly easier to design and synthesize the genes rapidly and directly which contains all possible alterations at specific sites that can enhance the thermostability of enzymes. In this regards, computer-based Gene Designer tool has been developed for *de novo* creation of genetic constructs. Additionally, Gene Designer can easily add, subtract, edit, modify, and combine genetic elements like tags, promoters, and open reading frames (Villalobos et al., 2006). These synthetic genes can be transcribed and translated in mainly two systems; first is cellular host systems and second is cell-free system (Lu, 2017).

4.1. Cell-systems for enzyme engineering

Currently, enzyme engineering relies on site-directed mutagenesis of gene in which a codon for particular amino acids is added or replaced. Basically, random mutagenesis and focused mutagenesis are used for this approach (Sunden et al., 2015). In addition to this, *de novo* synthesis of gene coding for thermostable enzyme can also be used. The modified gene is introduced into suitable cloning and expression system using recombinant DNA technology. Different cellular systems like *Escherichia coli*, *Bacillus* spp., and *Saccharomyces* spp. are most commonly used expression host for production of engineered enzymes. Several reports indicated the successful production of thermostable engineered enzymes in cell-systems. Irfan et al. (2018) has enhanced the thermostability of xylanase isolated from *Geobacillus thermodenitrificans* C5. They built six mutants of xylanase through site-directed mutagenesis by interchanging the residue with proline and glutamic acid. The mutants showed stability up to 75 °C which was 13 times more thermostable than wild-type xylanase. Similarly, in another study, the thermostability of recombinant phyA mutant phytase isolated from *Aspergillus niger* N25 was improved. The mutant enzyme showed significant improved catalytic efficiency and longest half-life after heat treatment (L. Tang et al., 2018; Z. Tang et al., 2018). In a study, the C-terminal region of nitrilase was engineered toward improvement of its thermostability. The mutant showed 14-fold higher stabilization at high temperature. Although, site-directed mutagenesis are most widely used for enzyme engineering but lower success rate, time-consuming, and high-cost are the major problems. Further, low protein yield, incorrect folding, expression variability, low stability, and insoluble expression limits its wider applications. The continuous advances in molecular biology tools for gene editing provides more effective platform for enzyme engineering for production of thermozymes.

4.1.1. Gene editing

Current researches are more focused towards gene editing tools like Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALEN), and Clustered regularly interspaced short palindromic repeats (CRISPR-Cas) for complete gene knock-in/knock-out or a small fragment of oligonucleotides knock-in/knock-out at single or multiple sites in host genome or site-specific mutagenesis (Dangi et al., 2018a). ZFNs have the potential to edit or cleave the target gene by specific type of zinc finger DNA-binding domain and introduce a nick in both strands of DNA using its endonuclease activity. However, several drawbacks like problems in designing, construction, low success rate, and high cost limits its application in gene editing (Kumar et al., 2016). On the other hand, TALEN, a special type of restriction enzymes are able to cleave the target DNA by using its TAL effector DNA-binding domain. The specific targeting is due to the presence of a simple "code" in conserved region of target sequence. TALEN has been successfully used for knock-out and knock-in of genes in the genome (Tanaka et al., 2018; L. Tang et al., 2018; Z. Tang et al., 2018; Xu et al., 2018). Similar to ZFNs, TALEN also possess some major limitations which need to be overcome. The CRISPR-Cas system is very complicated involving two crRNA, trans-acting antisense RNA (trcRNA), and a single CRISPR associated protein (Cas9). This above complex cleaves the specific target DNA. But off target cleavage is the major limitation of this system which also cleaves the non-target DNA at distant locations. Thus, researches are going on to overcome this off target effect of CRISPR-Cas system (Zhang et al., 2015). These gene editing tools create double stranded nick in target gene, which are further repaired by the error-prone non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR) using host repair machinery (Yadav et al., 2018).

4.2. Cell-free driven enzyme engineering

Enzyme engineering in cell-free system have the potential to overcome the limitations of cell-based system. Since it does not depend on cell growth, therefore synthesis can be directly controlled with lower complexity (Lee and Kim, 2013). In open system, enzyme engineering is much easier and can be performed in high throughput manner due to the direct access of the reaction environment used for enzyme synthesis. It allows real-time monitoring and manipulation of transcription and translation process. This system works using linear DNAs as templates for transcription which overcome the time consuming *in vivo* gene cloning and microbial transformation steps (Perez et al., 2016). Further, post-translation modifications and chemical conjugation is very convenient in cell-free system (Lu, 2017). As this system is in pioneering stage, thus most of the studies are carried out for cell-free system optimization. In this direction, different cell-free platforms derived from various cellular systems such as *Vibrio natriegens* (Des Soye et al., 2018), *E. coli* (Foshag et al., 2018), and *Pseudomonas putida* (H. Wang et al., 2018; J. Wang et al., 2018) are developed for protein synthesis. Recently, mammalian cell (CHO) lysate was used for the production of turbo green fluorescent protein (tGFP) and streptokinase enzyme (Tran et al., 2018). For enzyme engineering, this system involves incorporation of unnatural amino acids, enzyme synthesis and their proper folding, and seems promising for imparting thermostability.

4.2.1. Unnatural amino acids incorporation

In recent scenario, enzyme engineers are not limited with the addition of natural 20 amino acids for structural modifications to perform wide functions including stabilities under extreme conditions (Welegedara et al., 2018). They have developed different techniques to add unnatural amino acids (uAA) into enzymes and this addition is much easier in cell-free systems. This approach opens new avenue for designing enzymes with novel functions and improved properties. One approach to incorporate uAA in enzyme is to alter the genetic code through introduction of unnatural base pairs which will be transcribed in mRNA having unnatural bases resulting translation of polypeptide containing uAA (Zhang et al., 2013). Recently, researchers have designed aminoacyl-tRNA synthetases which have the ability to insert uAA at growing peptide backbone of enzyme (Crnković et al., 2016; Vargas-Rodriguez et al., 2018). This promising approach is yet to be tried for obtaining thermostable enzymes.

4.2.2. Enzyme synthesis and their proper folding

Enzyme engineering in cell-free system also includes high throughput enzyme synthesis and their proper folding which are difficult to accomplish in cellular or *in vivo* system. In cell-free system, multiple mRNA can be co-translated into enzyme. Thus, this process gives high yield of engineered enzyme without need of complex and multistep purification process (Lu, 2017; Zemella et al., 2015). Further, enzymes which require metals or other specific groups for their activity can also be synthesized in cell-free system easily. In addition to these, cell-free system eases the process of post-translation modification which is very difficult in cellular system for several enzymes (Carlson et al., 2012). Further, synthetic biology encourages the proper folding of enzymes to their native conformation by multiple disulfide bonds formation and high degree of hydrogen bonds which provides thermostability. This could be achieved by pre-treatment of reaction mixture with redox buffers, providing chaperones, and adding special disulfide bond-forming enzymes (Shimizu et al., 2005). One important model of enzyme-synthesis containing pre-defined cell-free reaction components is PURE (protein synthesis using recombinant elements) system which consist of 46 tRNAs, all corresponding enzymes, and 31 recombinant translational factors. This system was successfully employed for enzyme synthesis and incorporation of uAAs in enzyme (Lee and Kim, 2013). In this direction, *E. coli* derived customized cell-free extract devoid of amino acids degrading enzymes and endonuclease I was used for stable protein expression. The resulting cell-free systems produced protein with significant high yield (Michel-Reydellet et al., 2005). Thus, cell-free system as a synthetic biology tool would help in enzyme synthesis and proper folding to impart thermostability.

5. Recent trends in thermozymes applications

Microorganisms dwelling in environment with temperatures over 50 °C have been extensively contemplated, expanding the insight in synthesis and capacity of these natural networks. Microorganisms that inhabit in thermophilic environment produce thermozymes and their application through wide spectrum of biotechnology have the potential to be precious resources for the development of bio-based wealth. Analysis of biodiversity inside high thermal conditions is an area for dynamic research that constantly extends as innovation takes into account for novel methodologies and investigations. Also, their thermozymes, among different attributes, make thermophilic microorganisms of significant interest for their biotechnological potential (Berka et al., 2011). Industrial bioprocesses utilize thermophilic organisms for their potential repository of thermostable catalysts for various applications. They can be utilized in modern industrial procedures, in which they supplant enzymes from mesophiles (Bruins et al., 2001). Studies on thermophiles/hyperthermophiles are limited by the fact that their identification, isolation, and culturing strategies are cumbersome. To avoid this, high-throughput DNA sequencing has enabled the development and improvement of metagenomics. High-temperature ecosystems like hot springs (Ghelani et al., 2015; Gupta et al., 2012; López-López et al., 2015; Sangwan et al., 2015) have been analyzed using metagenomic approach. Therefore, metagenomics became an essential tool in recent years for the identification of thermozymes producing thermophiles from the microbial population in an environment. Cellulases from thermophiles (Escuder-Rodríguez et al., 2018), carbohydrate-related thermozymes (Kaushal et al., 2018), and other thermostable enzymes (Berini et al., 2017) have been sourced from non-culturable microbes employing metagenomic approach. With the advancement of next generation sequencing (NGS), more ecosystems can be

investigated at lower sequencing cost and time, enhancing the quality of 16S rRNA based biodiversity studies. NGS is enriched by captured metagenomics that involves the designing of custom hybridization-based oligonucleotide probes that hybridize with the metagenomic libraries followed by the sequencing of the probe-bound DNA fragments (DeCastro et al., 2016). Manoharan et al. (2015) has employed a GC independent strategy to avoid biasness in the detection of broader diversity of functional genes coding for carbohydrate-active enzymes and proteases in agricultural soils, this could also be an interesting tool to study thermophilic microbial populations.

Recent developments delineate the attributes of thermophiles for novel processes that are of extraordinary industrial applications e.g. amylases, pullulanases, xylanases, proteases, and cellulases are relied upon to assume a pivotal role in food, pharmaceutical, paper, pulp, and waste-treatment enterprises.

5.1. Biomass utilization

Thermozymes have been implicated extensively in industries by utilizing the microorganisms from time to time. Microorganisms harboring multienzyme complex "cellulosomes" on their cell surface which have been used from thermophilic anaerobes for delignification and carbohydrate degradation in industrial bioprocess for the generation of biofuel (Fontes and Gilbert, 2010). Recent developments in thermozymes explored the stability of designer cellulosomes at high temperatures *in vitro* and indicated expanded hydrolytic execution of thermophilic cellulosomes. Building a cellulosome producing thermophilic microorganism for the generation of biofuels is the favored system for the improvement of thermophiles (Moraïs et al., 2016). In an attempt to produce nanofibril cellulose, thermophilic enzymes such as endoglucanase, β-glucosidase, and α-amylase were used in combination with Star Burst System (SBS), a technology which works opposing water jet (Chaikaew et al., 2012). In a comprehensive study, Gupta et al. (2014) explained the utility of recombinant thermophilic enzymes from *Clostridium thermocellum* such as acetyl xylan esterase (AXE) and xylanases of glycoside hyrolase family (GH43) to saccharify the hemicelluloses of preprocessed *Populus nigra* and subsequent fermentation led to the higher yield of bioethanol. Thermophilic bacterial genera reported as source of efficient thermostable cellulolytic enzymes include *Clostridium*, *Bacillus*, *Acidothermus*, *Alicyclobacillus*, *Caldocellulosiruptor,* and *Thermotoga* (Yeoman et al., 2010). Several thermophilic endoglucanases have been isolated and cloned from genus *Clostridium*, CelE from *C. cellulolyticum* (Gaudin et al., 2000), Cel9 from *C. phytofermentans* (Zhang et al., 2010), Cel5 from *C. thermocellum* (Brumm et al., 2015), and CenC from *C. thermocellum* (Haq et al., 2015). Hyperthermostable endoglucanase had been studied from archaeal *Pyrococcus furiosus* and *Pyrococcus horikoshii*. Hyperthermophilic endoglucanases from *P. horikoshii* and *P. furiosus* belonging to GH family 5 and 12 respectively are highly thermostable, so these enzyme are expected to be useful for industrial applications (Kishishita et al., 2015). Recently, a novel technology has been developed to use graphene oxide treated thermostable crude cellulase to produce biohydrogen making use of sugarcane bagasse hydrolysate (Srivastava et al., 2018).

In addition, the thermozymes from fungal strains are also reported to have significant attributes in bioprocess industry. The fungal hemicellulase of *Mycothermus thermophilus* hydrolyzed alkali treated rice straw and bagasse into fermentable sugars efficiently. This could be used efficiently as industrial strain in biofuel industry (Basotra et al., 2016). *Rhizomucor* sp. and *Aspergillus* sp. among the fungal strains produced thermostable xylanase (active at 70 °C and stable up to 75 °C) under solid-state fermentation conditions (Robledo et al., 2016). Thermostable proteozymes also play a significant role in industry. Subtilisin, an endoprotease from *B. licheniformis* showed highest activity at 60 °C and pH of 8.3 (Synowiecki, 2008). These enzymes, active towards collagen, are useful for utilization of bones and some other wastes from meat industry. In other study, serine and metallo-proteases were isolated from *Bacillus stearothermophilus* and used in industrial waste activated sludge. Addition of divalent cations Ca^{2+} and Fe^{2+} ions increased the aerobic digestion of the sludge by the thermo-proteases (Kim et al., 2002). A thermotolerant, alkali-stable and oxidation-resistant protease (CHpro1) was found by functional screening of a metagenomic library of hot springs in Chumathang territory of Ladakh, India. This enzyme indicated optimal action at pH 11 and stability in high alkali range, which could be particularly intriguing for the cleanser business, as the pH of clothing cleansers is in the range of 9.0–12.0. The properties of this protease make it desirable for cleansing business (Singh et al., 2015). Amylopullulanase with thermophilic properties are indispensable in starch liquefaction and saccharification process. Thermostable amylopullulanase with potential applicability are reported from *Thermofilum pendens* (L. Li et al., 2018; X. Li et al., 2018), *Geobacillus thermoleovorans* (Nisha and Satyanarayana, 2017) and *Staphylothermus marinus* (Li et al., 2013). H. Wang et al. (2018) and J. Wang et al. (2018) cloned and expressed pullulanase (pulWB42), an important starch-debranching enzyme that is used in the food, chemical, and pharmaceutical industries from *Anoxybacillus* sp. WB42. Its catalytic properties above 60 °C make it an important candidate for use in starch bioprocessing. In another study a thermostable pullulanase was cloned and expressed from *Geobacillus thermocatenulatus* DSMZ730 which was used directly for maize starch saccharification without adjusting the pH, at reduced cost and improved efficiency (L. Li et al., 2018; X. Li et al., 2018). α-Amylase and glucoamylase are other indispensable thermozymes essential in starch processing industries. Bai et al. (2012) reported thermophilic α-amylase from *Alicyclobacillus* sp. functional optimum at 75 °C and pH 4.2 and showed 96.71% raw starch hydrolysis with commercial glucoamylase in a single step. Asoodeh et al. (2014) reported thermoacidophilic α-amylase from *Bacillus* sp. DR90 showing optimal activity at pH 4.0 and 75 °C and was marked prospective for food and detergent industries. α-Amylase from *Aspergillus oryzae* for maltose syrup production (Aleem et al., 2018) and commercial thermostable α-amylase for corn starch saccharification (Myat and Ryu, 2014) have been utilized successfully. Glucoamylase from *Aspergillus flavus* (Karim et al., 2016) and *Thermomucor indicae-seudaticae* (Kumar and Satyanarayana, 2003) are some representative examples for application in starch saccharification.

5.2. Food processing

Thermozymes are well characterized from many sources for their potential utilization in food processing industry. Thermophilic xylanases from compost fungus *M. thermophilus* CGMCC3.18119 cloned and expressed in *Pichia pastoris* displayed maximum activities at 65 °C. Controlled hydrolysis of beechwood xylan using the fugal xylanase produced varying degree of polymerized xylooligosaccharides which have high prebiotic potential and functional food activities (Ma et al., 2017). In another study, Sadaf and Khare (2014) employed solid-state fermentation of deoiled *Jatropha curcas* seed cake by *Sporotrichum thermophile* to produce themophilic xylanase, which hydolysed xylan polysaccharide to produce nutritive xylooligosaccharides. Functional attributes of thermozyme *endo*-β-(1 → 4)-mannanase active at 65 °C showed potential application in production of prebiotic mannooligosaccharides from commercial substrates and preprocessed copra meal (Ghosh et al., 2013). This study further showed that the mannooligosaccharides mannobiose and mannotriose produced from copra meal has better candidature as nutraceuticals and prebiotic activity (Ghosh et al., 2015). Table 2 illustrates some of the thermozymes deemed suitable to be used in industrial bioprocesses and food processing.

5.3. Environmental bioremediation

Due to their thermostability, current trend is to exploit them in environmental bioremediation of persistent organic pollutants. Further, low risk of contamination, higher substrate solubility and low viscosity

Table 2
Recent developments in thermophilic enzymes for industrial bioprocess and food processing.

Thermophiles	Corresponding thermozymes and Application	Operating temperature (°C)	Reference(s)
T. thermarum DSM 5069	Thermostable xylanase (Xyn10A) released higher sugar content by lowering the viscosity from steam treated biomass	85	Long et al. (2018)
Geobacillus sp. strain WSUCF1	Consortium of thermophile utilizes prairie cordgrass and produced biohydrogen	60	Bibra et al. (2018)
Acinetobacter pittii MASK 25	Production of xylopentose and xylohexose from agro biomass by the use of thermostable xylanase	50	Purohit et al. (2017)
Anoxybacillus sp. HBB16	Thermophilic novel alkaline lipase activity suitable for various industrial processes	55	Bakir and Metin (2017)
B. subtilis (TBS2)	Thermostable β-mannanase employed to hydrolyze mannan substrate to produce mannooligosaccharides for functional food	20–100	Luo et al. (2017)
Thermoanaerobacterium saccharolyticum	Engineered organism utilized to saccharify hemicelluloses into accessible sugars for generation of bioethanol	55	Herring et al. (2016)
T. thermarum DSM 5069	Thermostable β-glucosidase (TthBgl) released higher sugar content from cellobiose	85	Long et al. (2016)
T. thermophilus	The thermophilic bacterium *T. thermophilus* has been proposed as a good host for function-based detection of thermozymes	70	Leis et al. (2015)
Aspergillus fumigatus KIBGE-IB33	Higher production of glucose from cassava for further fermentation by *Saccharomyces* to achieve high ethanol conversion of 84%	65	Pervez et al. (2014)
S. thermophile	Thermophilc xylanase was produced from deoiled *Jatropha curcas* seed cake	45	Sadaf and Khare (2014)
Humicola insolens Y1	Three xylanase genes (xynA, xynB, xynC) of glycosyl hydrolase family 10 were identified and their biochemical properties were studied	70–80	Du et al. (2013)

make them better alternative in bioremediation. These enzymes can also be resistant to proteolysis and able to tolerate harsh conditions like presence of organic compounds, denaturing agents as well as high salinity (Raddadi et al., 2015). There are some well documented thermophilic microorganisms *viz. Caldicellulosiruptor*, *Sulfolobus*, *Thermotoga*, *Thermococcus*, and *Pyrococcus* genera that can produce wide array of thermozymes (Zeldes et al., 2015). Generally, mesozymes like oxidoreductases (oxygenases, laccases, peroxidases), and hydrolases (lipases, cellulases, carboxylesterases, phosphotriesterases, haloalkane dehalogenases) are used in biodegradation of pollutants (Dangi et al., 2018b; Sharma et al., 2018). The application of thermostable oxidoreductases and hydrolases in environmental bioremediation are still in pioneering phase so very few studies are available in this direction. Recently, Kadri et al. (2018) reported the production of thermostable alkane hydroxylase having optimum enzyme activity at 72 °C for the bioremediation of petroleum hydrocarbon like hexane, hexadecane, and motor oil. This enzyme was isolated from marine hydrocarbon degrading bacteria *Alcanivorax borkumensis*. In another study, potential thermostable enzymes like protease, amylase, phosphatase, ribonuclease, catalase, peroxidase, superoxide dismutase, and azoreductase having bioremediation potential were identified using LC–MS/MS from extracellular extract of *Anoxybacillus rupiensis* isolated form hot water spring in South Africa (Jardine et al., 2018). Similarly, thermo-alkali stable laccase having optimum temperature of 85 °C was produced from marine *Staphylococcus arlettae* S1-20 which was able to degrade ionic liquids (Chauhan et al., 2018). In another study, esterase was isolated from thermo-halophilic *Bacillus cereus* strain AGP-03 from hot spring of Bakreshwar, West Bengal, India. The enzyme showed thermostability at wide range of temperature (30–80 °C) and was found to be extremely tolerant for benzene (Ghati and Paul, 2015). Along with these, some of the other recently reported thermozymes having bioremediation potential are summarized in Table 3. The list illustrates their use in effective bioremediation, as an upcoming area of research.

6. Future perspectives

Thermozymes from extremely thermophilic microorganisms have been of industrial interest for quite a while due to their capacity to catalyze reactions at high temperatures. Genome and metagenome grouping information of extraordinary thermophiles give valuable data of putative biocatalysts for extensive variety of biotransformations (Zeldes et al., 2015). Control of protein stability is essential for understanding the rules that administer protein thermostability, both in fundamental research and industrial applications. Different information mining systems exist for the study of thermostable proteins. Besides, Artificial Neural Networking techniques have pulled in critical consideration for the prediction of thermostability, since they establish an appropriate way to deal with mapping the non-linear information yield connections (Rezaeenour et al., 2016). Extreme Learning Machine (ELM) approach can be applied to estimate thermal behavior of proteins from extremophiles by high throughput pattern analysis. In order to achieve the authentic patterns of nucleotide and amino acid sequences of thermozymes, proposed algorithm could be generated, under optimized parameters of ELM using a Genetic Algorithm (GA), which tuned a set of input variables, hidden layer biases, and input weights to enhance the prediction performance (Rezaeenour et al., 2016). Modern approach of genetic engineering, proteomics, and metabolic engineering aspects could play a significant role for understanding the paradigm of thermozymes functions for various industrial processes. Incorporation of nanotechnology and enzyme immobilization could be the useful technique for the use of thermozymes in industry. Reusability by enzyme immobilization shows several advantages for industrial applications (Shrivastava et al., 2012). Nanoparticles exhibit a number of unique characteristics as immobilization supports because of their elevated reaction surface. Additionally, magnetic nanoparticles, with an iron oxide core, covered by different types of polymers, allow an easy recovery of immobilized proteins. Although both materials proved to increase thermal stability of the enzyme, the latter had a better effect, showing the importance in the design and selection of immobilization supports. Interestingly, a report by Sadaf and Khare (2017) showed that the cells of *S. thermophile* can be used as a source of thermozymes predominantly a xylanase instead the use of crude enzyme leading to higher saccharification yield of wheat straw xylan. Therefore, future perspectives of modern industrial requirements of thermozymes can be enhanced by understanding the genetic toolkit of the thermophiles by high throughput analysis using computational biology, machine learning, artificial intelligence, neural networking, metabolic engineering, structural informatics, enzyme immobilization on nanoparticles, and ionic liquid stability of thermozyme for enhancement of catalytic efficiency as well as reusability.

7. Conclusions

Thermozymes continue to be in research focus on account of their

Table 3
Representative thermozymes used in bioremediation.

Enzyme	Source	Optimum temperature (°C)	Bioremediation	Reference(s)
Laccase	White rot fungi	50	Textile dyes	Yesilada et al. (2018)
Laccase	*Streptomyces ipomoeae* CECT 3341	60	Acid orange 63 dye	Blánquez et al. (2018)
Laccase	*Setosphaeria turcica*	60	2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)	Ma et al. (2018)
Phosphotriesterase-like lactonase	*S. acidocaldarius*	72	Organophosphates	Restaino et al. (2018)
Alkane hydroxylase	*Alcanivorax borkumensis*	72	Hexane and hexadecane	Kadri et al. (2018)
Carboxylesterase	*Pseudomonas syncantha* PS1	60	Pyrethroid pesticides	Cai et al. (2017)
Laccase	*Anoxybacillus* sp. strain UARK-01	90	Decolorization of congo red	Al-kahem Al-balawi et al. (2017)

robust nature and wide applicability in diverse sectors. A combination of structural features helps them to be stable at elevated temperatures. Continued research to source novel thermozymes from new isolates along with from metagenome will help to swell the available repository and application horizons of this class of enzyme. Further, synthetic biology tools can be employed to fine tune the properties of thermozyme to design them tailor made for specific application. Recent trends have shown their increased relevance in industrial bioprocesses and bioremediation. The research on them will continue to be in spotlight in coming years.

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Conflict of interest

The authors declare that they have no conflict of interest.

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